Mechanistic Influences for Mutation Induction Curves after Exposure to DNA-Reactive Carcinogens

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Abstract

A mechanistic understanding of carcinogenic genotoxicity is necessary to determine consequences of chemical exposure on human populations and improve health risk assessments. Currently, linear dose-responses are assumed for DNA reactive compounds, ignoring cytoprotective processes that may limit permanent damage. To investigate the biological significance of low-dose exposures, human lymphoblastoid cells were treated with alkylating agents that have different mechanisms of action and DNA targets: methylmethane sulfonate (MMS), methylnitrosourea (MNU), ethylmethane sulfonate (EMS), and ethylnitrosourea (ENU). Chromosomal damage and point mutations were quantified with the micronucleus and hypoxanthine phosphoribosyltransferase forward mutation assays. MNU and ENU showed linear dose-responses, whereas MMS and EMS had nonlinear curves containing a range of nonmutagenic low doses. The lowest observed effect level for induction of chromosomal aberrations was 0.85 μg/mL MMS and 1.40 μg/mL EMS; point mutations required 1.25 μg/mL MMS and 1.40 μg/mL EMS before a mutagenic effect was detected. This nonlinearity could be due to homeostatic maintenance by DNA repair, which is efficient at low doses of compounds that primarily alkylate N^7-G and rarely attack O atoms. A pragmatic threshold for carcinogenicity may therefore exist for such genotoxins. [Cancer Res 2007;67(8):3904–11]

Introduction

Cancer has been closely associated with the variety of carcinogens that humans come into contact with, not only in their natural environment but also through occupational and recreational exposures. Assessing and characterizing the mechanisms of such agents in promoting genetic damage is therefore important in gaining a more complete understanding of their carcinogenic potential. It has been assumed that a linear relationship exists between exposure to DNA reactive genotoxins, the induction of DNA lesions, and their conversion to mutagenic alterations (1, 2). However, mammalian cells have a number of homeostatic mechanisms *in vivo* that provide protection to a certain extent (e.g., DNA repair, chemical detoxification, or a redundant target). These mechanisms initially prevent damage induced from becoming a permanent defect until they become saturated, thus resulting in a no observed effect level (NOEL), the experimental concentration below which no statistically significant increase in mutations is detected. However, the existence of such a threshold effect for agents with carcinogenic potential has been long debated and is still controversial. Currently, there is only limited data with regard to the kinetics of mutation induction in the low-dose region.

Non–DNA reactive agents have been shown to demonstrate NOELs (3, 4), primarily due to the redundancy of their cellular targets. Aneugenic compounds, such as colchicine, nocodazole, vinblastine, and griseofulvin (5–7), that inhibit tubulin polymerization are prime examples. As the spindle apparatus consists of many tubulin monomers, its inactivation would require multiple targets to be damaged before a significant adverse effect occurs; thus, chromosome segregation is only altered at the concentration that prevents the formation of sufficient microtubules. In contrast, DNA reactive chemicals have been assumed to have a non-threshold mode of action, as they directly induce DNA lesions that have the potential to be fixed as point mutations or chromosomal aberrations. However, homeostasis in mammalian cells allows them to adapt to environmental insults, which may be predicted to limit genetic damage, therefore introducing a range of low doses that have biologically insignificant effects (i.e., NOEL). Determining the existence of threshold dose-responses for genotoxic compounds has important implications because exposure to low concentrations within the NOEL would carry little carcinogenic risk as DNA aberrations are not fixed, thus they have no biological significance or further consequences on the cell. Conversely, in the case of chemotherapeutic agents, ensuring that treatment doses fall above any threshold is essential for efficient tumor killing.

An important class of DNA reactive genotoxins also used as chemotherapeutics are alkylating agents (8). Their modes of action and DNA targets have been well characterized, and they include a wide variety of chemicals, many of which are suspected or proved carcinogens. Human exposure to alkylating agents is frequent in daily life through occupational sources, medical treatments, and several endogenous sources, including nitrosation of compounds in food and tobacco smoke into agents that alkylate DNA (9–11). Alkylating agents can be classified as monofunctional or bifunctional (i.e., single or double reactive groups), and they are electrophilic; thus, they react with electron-rich nucleophilic centers within DNA and proteins, with their specific targets depending upon the reactivity of the individual chemicals. The Swain Scott constant (*s* value) is used as a standard measure of the reactivity of alkylating agents (12). Compounds with high *s* values target highly nucleophilic centers (e.g., N^7-G, N^3-A), whereas low–*s* value alkylating agents are less sensitive to the level of nucleophilicity of their target; thus, they do not discriminate between centers of high and low nucleophilicity and so are able to alkylate O atoms (e.g., O^6-G) much more efficiently than those with high *s* values.

Conflicts of interest: The authors have no conflicts of interest.

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To understand the mutagenic potential of DNA reactive compounds, we need to determine the mechanisms which influence the relationship between exposure, DNA adduct formation, and consequent genetic damage. Humans are most frequently exposed to monofunctional alkylating agents; thus for the present study, we have chosen to use model compounds with similar adduct spectra to those humans we come into contact with. Four model monofunctional alkylating agents with differing mechanisms were therefore used to assess the biological significance of low-dose exposures: methylmethane sulfonate (MMS) and ethylmethane sulfonate (EMS) that primarily induce N^7-G adducts and methylnitrosourea (MNU) and ethylnitrosourea (ENU) that potently induce O adducts.

The alkylsulfonates were initially developed as chemosterilants for insects and mammalian pests (13). MMS has been used as a solvent, insecticide, and chemotherapeutic agent, but due to the strong carcinogenic potential of this group of agents, both MMS and EMS are now primarily used as model alkylating agents. MMS and EMS have high *s* values, thus react predominantly with ring N atoms (Table 1; ref. 14), and consequently both are potent mutagens due to mispairing during replication or the production of abasic sites and single-strand DNA breaks (15). The nitrosoureas have been used in the synthesis of diazo compounds, but again both MNU and ENU are highly mutagenic (13). Nitrosoureas are much more potent mutagens than methanesulfonates, and they also differ in their preferred DNA target sites. Most alkylating agents target the N^7 and, to a lesser extent, the O^6 positions of guanine, but MNU and ENU have high affinity for oxygen atoms due to their low *s* values (Table 1; ref. 13). In addition, they are carbamoylating agents, which involve the transfer of their alkyl groups to nucleophilic amino groups within nuclear proteins (16). Generally, methylating agents are ~10-fold to 20-fold more reactive than ethylating agents, as the larger groups are less efficiently transferred. Ethyl adducts are considered more mutagenic, probably as methyl adducts are repaired with greater efficiency (13, 17). However, factors, such as alkylating agent reactivity and the sequences they preferentially target, must also be taken into account.

Table 1. DNA adduct profiles for MMS, MNU, EMS and ENU

Adduct	MMS	EMS	MNU	ENU
s value	>0.83	0.67	0.42	0.26
N^7-G	81–83	58–65	65–70	11–11.5
N^3-G	0.6	0.3–0.9	0.6–1.9	0.6–1.6
N^7-A	1.8	1.1–1.9	0.8–2	0.3–0.6
N^3-A	10.4–11.3	4.2–4.9	8–9	2.8–5.6
N^3-T	0.1	Nd	0.1–0.3	0.8
N^3-C	<1	0.4–0.6	0.06–0.6	0.2–0.6
O^6-G	0.3	2	5.9–8.2	7.8–9.5
O^2-T	Nd	Nd	0.1–0.3	7.4–7.8
O^4-T	Nd	Nd	0.1–0.7	1–2.5
O^2-C	Nd	0.3	0.1	2.7–2.8
Phosphotriesters	0.8	12–13	12–17	55–57

NOTE: Adapted from Beranek (13). Data are in percentages; all possible adducts were not included, so columns do not add up to 100%.
Abbreviation: Nd, not detected.

We have, therefore, focused on two pairs of model alkylating agents, MMS/MNU and EMS/ENU, to investigate low–dose-response relationships after exposure to DNA reactive genotoxins, assessing the induction of chromosomal damage and gene mutation. These compounds were chosen due to their differing reactive mechanisms, DNA targets, and sizes of alkyl groups. In addition, their adducts are removed, at least in part, by different repair mechanisms. Thus, comparisons both between and within these groups allow us to relate the consequent results to their mechanisms and infer if repair is involved. Hence improving our understanding of the underpinning factors that contribute to nonlinear dose-responses and, thus, govern the mutagenic potential of this group of carcinogens.

Materials and Methods

Chemicals. MMS, MNU, EMS, and ENU were all purchased from Sigma (Dorset, United Kingdom). All chemical dilutions were freshly prepared from stock solutions with water.

Cell culture. The human lymphoblastoid cell line AHH-1 (18) was cultured in RPMI 1640 (Life Technologies, Paisley, United Kingdom) supplemented with 1% L-glutamine (Life Technologies) and 10% donor horse serum (BDGentest, Oxford, United Kingdom). The cells were maintained at a concentration of 1 to 2×10^5/mL.

Cytokinesis blocked micronucleus assay. AHH-1 (10 mL) suspension with cells at 1×10^5/mL were seeded for 24 h at 37°C, 5% CO_2. Each was dosed with appropriately diluted test chemical (in duplicate) and 3 μg/mL cytocholasin B for one cell cycle. Treated cells were harvested, cytospun onto polished glass slides, fixed in 90% methanol, stained with acridine orange (12.5 mg/100 mL phosphate buffer, pH 6.8), and viewed under an Olympus BX50 fluorescence microscope with a UplanF1 100× per 1.3 oil objective. The criteria for identifying micronuclei were as previously described (19). Two thousand binucleated cells were scored per treatment (1000 per duplicate). However, the number of binucleate cells that were analyzed was increased to 10,000 for several doses surrounding the NOEL.

Kinetochores were stained to classify test chemicals as clastogenic or aneugenic with CREST antibody (Quadratech, Surrey, United Kingdom). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole, and 100 micronuclei per dose were scored as positive or negative for kinetochore(s) under an Olympus BX50 fluorescence microscope.

Forward mutation assays. To remove background mutants within the AHH-1 cell population, 5×10^5 cells/mL were grown for 3 days in the 2×10^{-4} mol/L hypoxanthine–8×10^{-7} mol/L aminopterin–3.5×10^{-5} mol/L thymidine (HAT) media, followed by 24 h in HT media (as HAT media but without the aminopterin) and then transferred to normal growth media for 3 to 4 days. Cell suspension (10 mL) at 5×10^5/mL were exposed to the test chemical at the appropriate concentration, for 24 h at 37°C, 5% CO_2. The cells were washed to remove the alkylating agent resuspended in 50-mL fresh media and incubated for 13 days. Then the 96-well plates were loaded with 4×10^4 cells per well in selective media containing 0.6 μg/mL 6-thioguanine [hypoxanthine phosphoribosyltransferase (*HPRT*) assay] or 4 μg/mL trifluorothymidine [thymidine kinase (*TK*) assay]. In addition, plates without selection containing 20 cells per well were also required to determine the plating efficiency. All plates were incubated for 14 days at 37°C, 5% CO_2, in a humidified atmosphere. Subsequent colony formation (>20 cells in diameter) was scored to determine the mutation frequency of each dose, calculated as described by Furth et al. (20).

For each dose, 20× 96-well plates for assessing mutation frequency and another 20 for plating efficiency were set up. However, this was increased to 100 mutation frequency and 50 plating efficiency of 96-well plates for eight doses surrounding a potential NOEL.

Statistical analysis. A one-way ANOVA, followed by a Dunnett's posthoc test, was used to determine if any of the treatment doses were significantly different from the zero dose. Statistical modeling was done by root transformation of the data once the control values had been subtracted $[\sqrt{(\{x - x_c\} + 0.5)}]$ to standardize it, followed by plotting a log graph to determine the NOEL/lowest observed effect level (LOEL) concentrations.

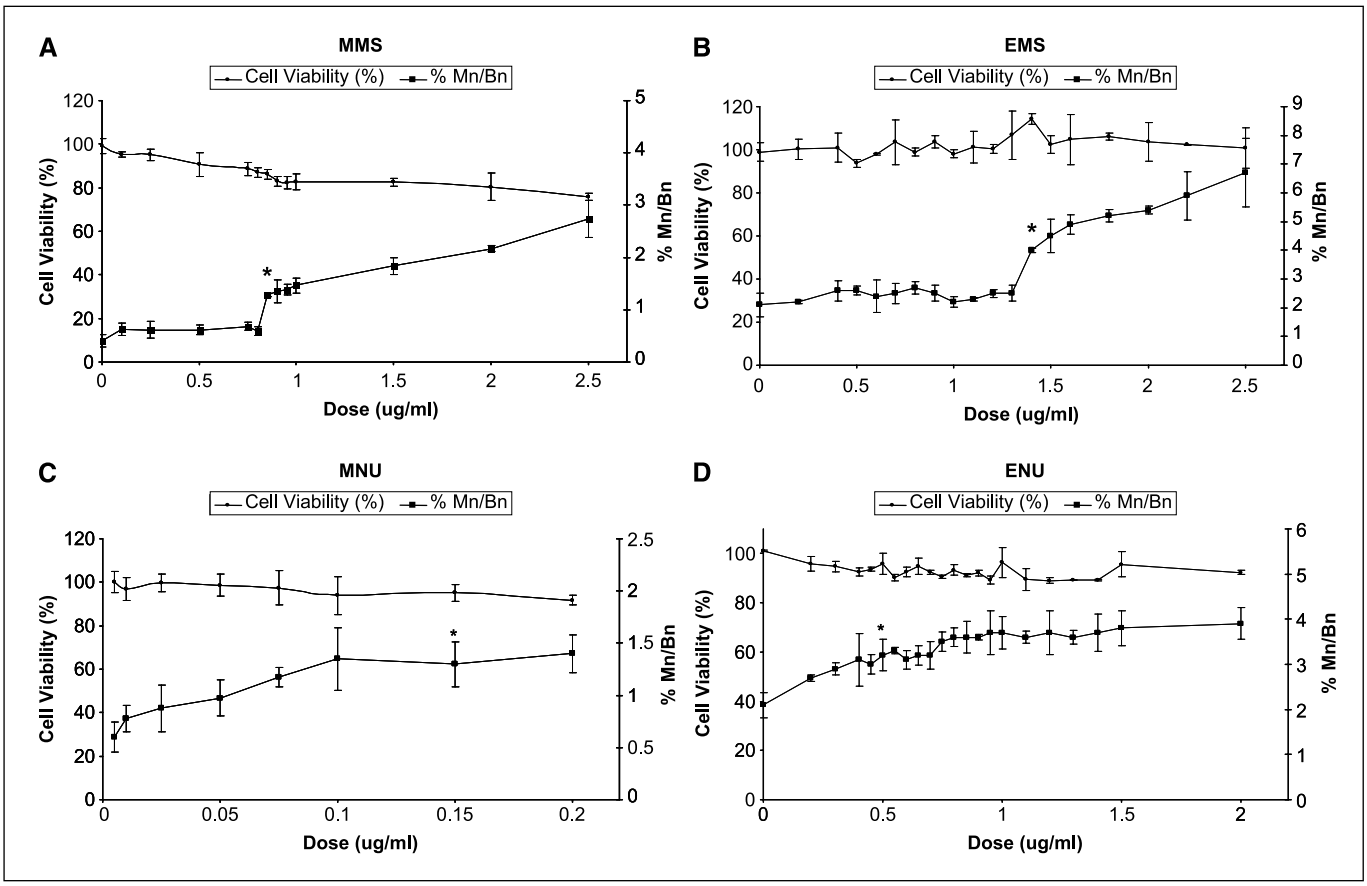

Figure 1. Influence of MMS (*A*), EMS (*B*), MNU (*C*), and ENU (*D*) dose upon micronucleus frequency in the AHH-1 cell line. *Points,* mean of treatments done in duplicate; *bars,* SD. *, the first statistically significant increases in chromosome damage at 0.85 μg/mL MMS (*A*), 1.40 μg/mL EMS (*B*), 0.15 μg/mL MNU (*C*), and 0.50 μg/mL ENU (*D*); *%Mn/Bn,* percentage of binucleated cells containing one or more micronuclei.

Results

The cytokinesis blocked micronucleus (CBMN) and *HPRT* forward mutation assays were used to assess the frequency of chromosomal damage and point mutations, respectively, after exposure to low concentrations of MMS, MNU, EMS, and ENU, to determine whether we can estimate NOEL and LOEL values, and thus, whether there are nonlinear dose-response relationships for these model alkylating agents. Initial dose setting assays were done for each compound, with both assays to identify the low-dose range of the concentration-response curves (data not shown). Subsequently, doses ranging from 0 to 2.5 μg/mL and 0 to 2.0 μg/mL MMS, EMS, and ENU were focused on for further detailed analysis in the CBMN and *HPRT* assays, respectively. For MNU, the corresponding dose ranges assessed were 0 to 0.08 μg/mL and 0 to 0.2 μg/mL.

Chromosomal aberration induction. The dose-response relationships of MMS, MNU, EMS, and ENU, with respect to the induction of chromosome damage, are illustrated in Fig. 1. A NOEL ranging between 0 to 0.80 μg/mL MMS and 0 to 1.35 μg/mL EMS was identified. Very sharp and statistically significant increases in micronuclei frequency were then observed, resulting in LOEL concentrations at 0.85 and 1.40 μg/mL for MMS and EMS, respectively ($P < 0.05$). Subsequent increases in concentration above the LOEL resulted in progressive increases in chromosomal damage, as detected by increasing frequency of micronuclei formation.

The alkylnitrosoureas, however, exhibited very different dose-responses compared with the alkylmethanesulfonates. No clear NOEL was observed with either MNU or ENU (Fig. 1), instead with each increase in dose, there was an incremental increase in micronucleus induction. The first dose that induced a statistically significant positive response was 0.15 μg/mL MNU and 0.50 μg/mL ENU ($P < 0.05$), although increases in the micronucleus frequency above the control were observed at lower doses in both cases.

Over the dose ranges assessed, the methylating agents induced reductions in cell viability. At the top dose of 2.5 μg/mL MMS, cell viability was at 76%. MNU was the most potent alkylating agent, demonstrating only 64% cell viability at 1.5 μg/mL. In contrast, the ethylating agents had a more moderate effect on cell survival over the experimental concentrations. Despite some fluctuations, EMS had very little effect on lowering cell viability between 0 and 2.0 μg/mL, whereas ENU resulted in a slight reduction to 90% at 2.0 μg/mL. Thus, overall the order of toxicity is MNU > MMS > ENU > EMS. However, for all four alkylating agents, LOEL was at concentrations that did not severely diminish cell survival at 90% to 100% viability. Thus, the genetic damage induced at these doses was not a consequence of cytotoxicity-related mechanisms.

Kinetochore staining was subsequently used to determine whether the micronuclei induced by the alkylating agents contained whole chromosomes or fragments. In all cases, as dose

increased the proportion of kinetochore, negative micronuclei increased, thus confirming that these compounds predominantly act via clastogenic mechanisms, inducing chromosome breakage and not aneuploidy.

***HPRT* mutation frequencies.** To obtain sufficient statistical power, 100× 96-well plates per dose with 4×10^4 cells per well were scored for each concentration analyzed within the low-dose ranges to be characterized. Figure 2 illustrates the mutation frequencies for each dose assessed with all the four test compounds.

Both MMS and EMS showed a range of low doses that lay within a NOEL, 0 to 1.0 and 0 to 1.2 μg/mL, respectively. A sharp increase in mutation frequency was then observed at 1.25 μg/mL MMS and 1.4 μg/mL EMS, which represented their LOEL. A 3-fold and 5-fold increase in mutation frequency was therefore observed between the NOEL and LOEL doses for MMS and EMS, respectively. Statistical modeling was done by root transformation of the data once the control mutation frequency had been subtracted to normalize the data before analysis. This method focuses on the NOEL and LOEL concentrations to determine if they are significantly different from the control values. Indeed, the statistical analysis confirmed that NOEL doses did not significantly differ from the control, whereas LOEL doses represented the first statistically significant increase in mutation frequency observed for both MMS and EMS ($P < 0.05$), thus demonstrating that the alkyl methanesulfonates have nonlinear dose-responses.

In contrast to the MMS and EMS results, and in agreement with the CBMN assay data, MNU and ENU show linear dose-responses even when very low concentrations are used (Fig. 2). The first significant ($P < 0.05$) increase in mutation frequency occurred at 0.0075 μg/mL MNU and 0.4 μg/mL ENU, with no obvious NOEL and simply a steady increase in mutation frequency with increasing dose. Consequently, the order of mutagenicity for the four model alkylating agents assessed in human lymphoblastoid cells was MNU > ENU > MMS > EMS. By reviewing Table 1, we can speculate that this order may be directly associated with the levels of O^6-alkyl-G adduct induction, particularly as EMS and MNU induce very similar levels of all adducts with exception of the O^6-G site, yet the latter is a far more potent mutagen.

***HPRT* assay validation.** To assess the mutation frequency in a different gene locus, the experiment with MMS was repeated with the *TK* forward mutation assay. The resultant *TK* dose-response curve was very similar to the *HPRT* data (Fig. 3), with a NOEL observed between 0 and 1.0 μg/mL MMS and a significant LOEL at 1.25 μg/mL MMS in the *TK* assay. The *TK* locus was generally found to have a higher background mutation frequency than *HPRT* (~10-fold), but this is the standard difference between the two loci and is due to the high proportion of mutations at the *HPRT* locus being lethal. As both the *HPRT* and *TK* loci showed the same NOEL and LOEL concentrations after treatment with MMS, it suggests a genome-wide increase in mutation frequency after exposure to the alkylating agents through the same mutagenic mechanism.

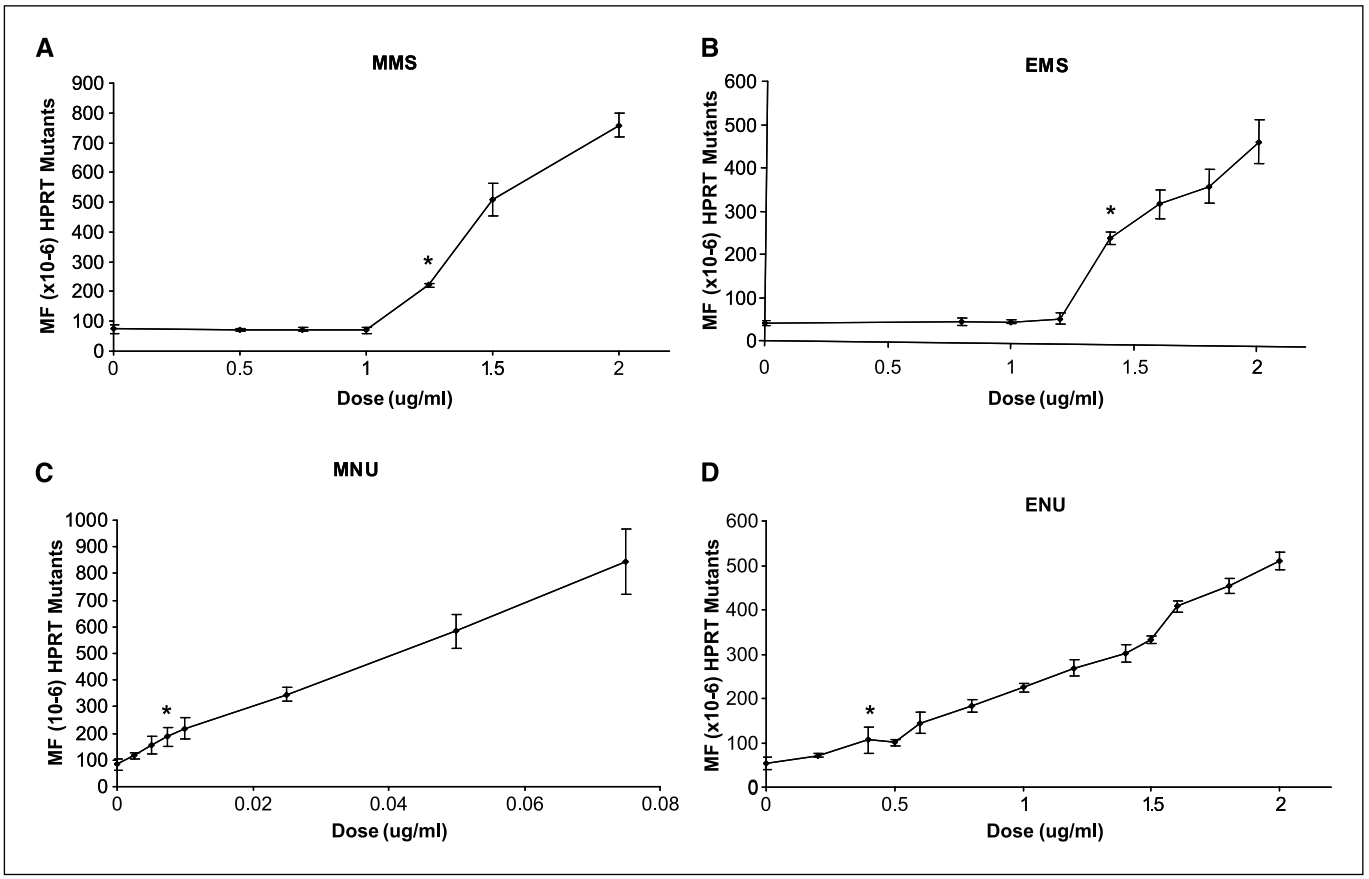

Figure 2. Dose-response relationships of MMS (*A*), EMS (*B*), MNU (*C*), and ENU (*D*) with respect to *HPRT* gene mutation frequency (*MF*). *, the first statistically significant increases in mutation frequency at 1.25 μg/mL MMS (*A*), 1.40 μg/mL EMS (*B*), 0.0075 μg/mL MNU (*C*), and 0.40 μg/mL ENU (*D*); *MF,* number of 6-thioguanine resistant clones/10^6 clone-forming cells. *Points,* average mutation frequency calculated from 100× 96-well plates, each dose done in triplicate; *bars,* SD.

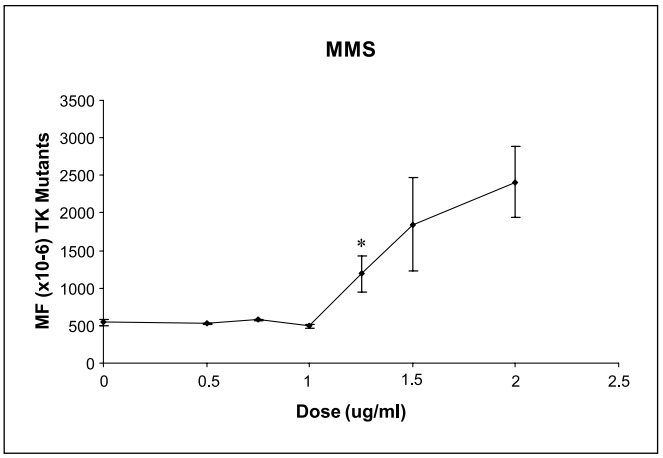

Figure 3. MMS mutation frequency dose-response relationship according to the TK forward mutation assay. *MF,* the number of trifluorothymidine resistant clones/10^6 clone-forming cells. *Points,* average mutation frequency calculated from 100× 96-well plates, with each dose done in triplicate. *, the first statistically significant increase in mutation frequency at 1.25 μg/mL MMS.

In addition, it is possible that the observed NOEL/LOEL response was limited by the sensitivity of the *HPRT* assay rather than the NOEL for mutation induction by the alkylating agents. As the number of cells treated per experiment governs assay sensitivity, in addition to our standard assay (using 4×10^5 cells/mL), the MMS experiment was repeated with cells plated in selective media at a concentration of 2×10^5/mL and 6×10^5/mL. As Fig. 4 illustrates, an increase in overall mutation frequency was observed due to the same concentration of selective toxic analogue with an increased number of cells plated, but the position of the NOEL and LOEL doses remained the same regardless of the starting concentration of cells. Consequently, the assay used is considered sensitive for analysis of mutation frequency in these low-dose regions, as true dose-responses were evident.

Discussion

Characterization of the genotoxic mechanisms of xenobiotic compounds is important for human health risk assessment and in understanding their carcinogenic potential. DNA reactive agents have been shown to induce DNA adducts in a linear manner with increasing dose (21–23). Consequently, such compounds are currently assumed to have linear dose-responses and abide by the "single hit, single target" hypothesis, whereby a mutation (that has the potential to promote neoplastic transformation) results from a single interaction, causing a nucleotide base change (24). However, this does not take into account the cytoprotective mechanisms that potentially counteract any disturbances induced after exposure to genotoxic agents, thus limiting the abnormalities observed. We therefore did a quantitative analysis with four alkylating agents (demonstrating different modes of action and DNA targets) as model compounds to investigate the biological significance of low-dose exposures. Hence, a simple *in vitro* system based upon a human cell model was used to assess intrinsic cellular defense.

Consistent with popular belief, MNU and ENU displayed linear dose-responses in both assays used for this investigation. The lowest doses that induced a statistically significant increase in genetic damage were 0.15 μg/mL MNU or 0.5 μg/mL ENU for chromosomal damage and 0.0075 μg/mL MNU or 0.4 μg/mL ENU for the induction of point mutations. Although increases in genetic damage were apparent at lower doses, they lacked significance but they indicate that the presence of MNU and ENU at any level has the potential to generate a detectable adverse effect.

However, strikingly, we have showed that some compounds that interact directly with DNA to form adducts have a nonlinear dose-response. MMS and EMS both display a range of low doses that represent a NOEL, with LOELs at 0.85 μg/mL MMS and 1.4 μg/mL EMS for the induction of chromosomal damage, and LOELs at 1.25 μg/mL MMS and 1.4 μg/mL EMS for point mutations. The LOELs were not at concentrations that significantly decreased cell viability; thus, the sharp increases in chromosomal damage and mutation frequencies observed were not induced via a cytotoxicity-related mechanism. This lack of cell death produces viable mutant cells which correlates with increased concern for tumorigenic potential *in vivo*.

With respect to both the induction of chromosomal damage and point mutations, the order of potency of the test compounds was MNU > ENU > MMS > EMS, which is in agreement with the literature (25), and might be associated with the level of O^6-alky-G adduct induction (Table 1). However, the lesions that cause gene mutations are different from those that cause chromosomal breaks.

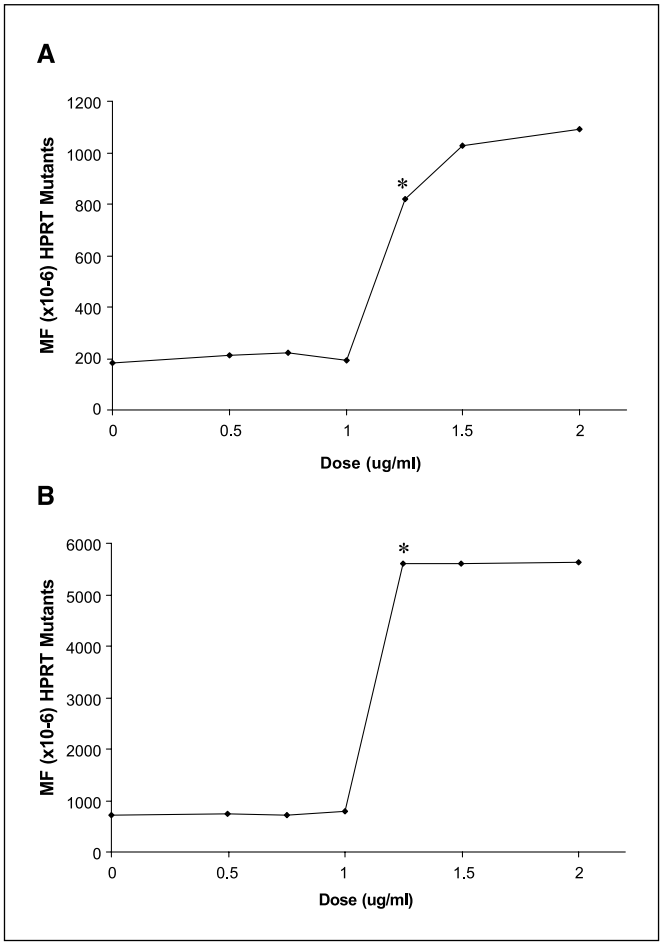

Figure 4. HPRT mutation frequency induced by MMS with differing cell plating concentrations for assay validation. *A,* 2×10^5 cells/mL; *B,* 6×10^5 cells/mL. In both cases, a significant LOEL is evident at 1.25 μg/mL MMS (*), indicating that nonlinearity is a true dose-response and not a consequence of assay sensitivity.

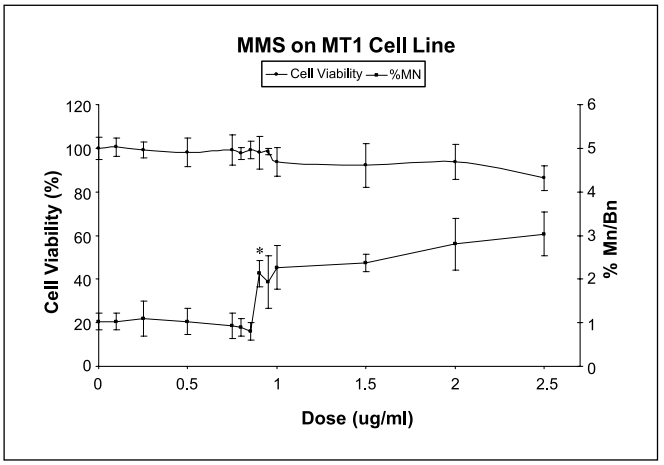

Figure 5. Influence of MMS dose upon micronucleus frequency in the MGMT deficient MT1 cell line. *, the first statistically significant increase in chromosome damage at 0.85 μg/mL.

O^6-alky-G, O^2-alkyl-T, and O^4-alkyl-T are directly associated with the induction of GC to AT, AT to TA, and AT to GC point mutations, respectively, whereas alkylation at N atoms are thought to be the dominant precursors for chromosomal damage (26, 27). Consequently, the NOELs observed in the CBMN assay with MMS and EMS treatments are likely to be due to the repair of N^7-alkyl-G, whereas repair of O^6-alky-G could give rise to the NOELs displayed with these compounds in the *HPRT* assay. Phosphotriesters are also a frequent target for alkylation, particularly with ENU (Table 1). They are believed to interfere with DNA-protein binding but do not seem to inhibit replication (28). In addition, there is no evidence to suggest that alkyl-phosphotriesters are premutagenic lesions; thus, they are unlikely to contribute to the dose-response relationships observed in the current study.

There are a number of mechanisms, including detoxification, conjugation, and exclusion, that may be responsible for contributing to genotoxic thresholds, particularly in the *in vivo* situation (11). However, DNA repair is the putative mechanism that is thought to be responsible for biological pragmatic threshold dose-responses after exposure to genotoxic compounds, as it is primarily responsible for fixing DNA adducts as permanent damage and may well be able to successfully remove such adducts at low levels (29, 30). Thus, at low doses, the rate of adduct repair is faster than its rate of formation, resulting in a NOEL. The key repairable adducts induced by alkylating agents occur at the N^7 and O^6 positions of guanine, and they are corrected via different mechanisms. N^7-alkyl-G adducts are substrates for the base excision repair (BER) pathway (9) and are removed to a lesser extent by mismatch repair (31), whereas the methylguanine DNA methyltransferase (MGMT) enzyme is primarily responsible for repair of O^6-alkyl-G adducts, although nucleotide excision repair is also thought to be involved occasionally when the MGMT enzyme is overloaded (9, 27).

When the alkylmethane sulfonates are compared with their counterpart, the nitrosoureas (Table 1), they generally induce far more N^7-G adducts, considerably less O^6-G adducts, and do not induce thymine adducts at all. At low doses, BER may be responsible for removing the N^7-alkyl-G lesions induced by MMS and EMS, whereas the MGMT enzyme can probably cope with the low levels of O^6-alkyl-G adducts, thus resulting in their NOEL concentration ranges for the induction of chromosomal damage and point mutations, respectively. In support of this theory, we have preliminary data demonstrating that expression of the *MGMT* gene is significantly up-regulated when cells are treated with MMS at doses below the pragmatic threshold (data not shown). The increased quantity of repair enzyme is likely to be responsible for removing adducts and therefore preventing mutagenic change. However, at the LOEL concentrations, these repair pathways are working at their full capacity; adducts will start to escape repair, become fixed permanent defects, and subsequent increases in dose result in a more linear increase in damage. The MMS and EMS NOELs that we have shown experimentally may therefore be due to efficient repair by both BER and MGMT.

MNU and ENU also induce N^7-alkyl-G adducts that may be efficiently repaired by BER at low doses, but they also induce a comparatively larger proportion of O^6-alkyl-G lesions that may be too extensive for the MGMT enzyme to correct even at low doses. In addition, they induce adducts at O^2-T and O^4-T, which are both very poorly repaired; thus, the unrepaired O adducts may result in their linear concentration-responses. The importance of the thymine adducts have been shown by Guttenplan (32) when he reported that, at low ENU doses, mutations at AT sites predominated (as a result of O^2-ethyl-T and O^4-ethyl-T), but as concentrations increased, more mutations were induced at GC sites due to saturation of the MGMT enzyme, and thus, persistence of O^6-ethyl-G. MNU has a very similar adduct profile to EMS, but it induces a higher proportion of adducts at O atoms; thus, the linearity the MNU shows may be due to the excess of O^6-methyl-G that are not completely removed and the unrepaired O^2-T and O^4-T adducts. Consequently, the low level of O^6-alkyl-G induced by MMS (0.3%) and EMS (2%) at concentrations below 1 μg/mL and 1.4 μg/mL, respectively, could be coped with, whereas higher proportions of 6% to 9% (induced by MNU) likely saturate the protective mechanisms, leading to increasing mutation levels. There is a possibility that adduct profiles may be dose dependent, and thus, at low doses, adduct proportions may differ from those detailed in Table 1. However, at present there is no data to support this theory.

To determine if repair is indeed responsible for the differing dose-responses and to distinguish which adducts are responsible for the NOELs, these experiments need to be repeated in repair-deficient cell lines, in which more linear dose-responses or lower LOEL concentrations would be expected. We now have some preliminary data indicating that the position of the NOEL/LOEL values, with respect to chromosomal damage, remained unchanged when the MGMT deficient cell line MT1 is treated with MMS (Fig. 5). This provides further evidence to suggest that repair of N^7-alkyl-G adducts are responsible for the NOEL, in which chromosome damage is concerned. However, we consider that nonlinear dose-response is likely to be displaced when this experiment is repeated with the *HPRT* assay, as the point mutations induced are mostly due to O^6-alkyl-G lesions. In repair proficient cell lines, repair, particularly by the MGMT enzyme, may be removing O^6-alkyl-G adducts at low doses, giving rise to the nonlinear dose-responses we have shown for MMS. This activity is absent in the MT1 cell line; thus, we would expect a reduced NOEL range with the *HPRT* assay due to the reduced efficiency in the removal of O^6-alkyl-G, leading to an increased level of point mutation induction.

It is of importance to note that the differences in mutagenicity at equal exposure levels are not purely due to adduct profiles. The reactivity of the alkylating agents toward DNA is also important

and is governed by the compounds' physiochemical properties, including the migratory rate of the chemicals through the cell, rate of alkylation or hydrolysis and their half-lives, factors which all control the overall number of adducts formed. For example, Table 1 indicates that a higher proportion of adducts induced by ENU is at O^6-G compared with MNU, but at equimolar doses MNU is the more potent mutagen. This is probably caused by the higher reactivity that MNU shows toward DNA compared with ENU (16, 33). Consequently, MNU has been shown to induce a higher frequency of DNA adducts than equimolar concentrations of ENU (34). Furthermore, MNU preferentially targets internucleosomal regions and transcribed sequences (35, 36), whereas ENU has a higher affinity for chromosomal proteins (16). Thus, chemical reactivity governs mutagenicity, whereas O^6-alkyl-G levels may be responsible for the shape of the dose-response curves.

It is recognized that one of the primary issues in defining a nonlinear dose-response is the sensitivity of the assay. To overcome this, we increased the typical number of cells scored in both test systems by a factor of 5; thus, 10,000 binucleated cells were examined in the CBMN assay and 100× 96-well plates containing 4×10^4 cells per well were used in the forward mutation assays. Consequently, the techniques used were highly sensitive and robust, with high statistical power. Furthermore, both *HPRT* and *TK* forward mutation assays resulted in the same NOEL and LOEL values for MMS (1 and 1.25 μg/mL, respectively) indicating these values do not change between assays. In addition, the different dose-response curves after EMS and ENU treatment with the *HPRT* assay has been previously reported *in vivo* (37). The nonlinearity of EMS was also attributed to the efficient removal of O^6-ethyl-G at low levels by MGMT, whereas poor repair of thymine adducts explained the linear dose-response of ENU, as mutations were primarily located at AT sites.

The existence of a NOEL implies at least a pragmatic threshold for carcinogenicity of these genotoxic agents. Theoretically, as genetic changes do not arise at very low doses (within the NOEL), carcinogenesis may be unlikely to occur after exposure to concentrations below the LOEL, as no biologically significant effects are observed experimentally. This outcome also has important implications in patient responses to chemotherapeutics involving alkylating drugs, particularly as MMS-based drugs are in use in addition to other methylating agents, such as procarbazine and dacarbazine, which have similar adduct profiles to MMS (38). The therapeutic dose would have to be significantly above the LOEL to maximize the cytostatic effects of the drug on cancer cells. Alternatively, if repair is responsible for the nonlinear dose-responses, then perhaps drugs that inhibit repair in the treated cells are also required to enhance the effects of the cytotoxic drugs at low concentrations.

We have therefore shown a NOEL for genotoxicity induced by MMS and EMS, which indicates that mammalian cells can withstand exposure to low concentrations of certain genotoxic agents and maintain a stable genome possibly due to physiologic protective mechanisms, such as DNA repair. Current regulatory opinion holds that all DNA reactive genotoxins abide by the "single hit, single target" hypothesis, whereby a mutation may result from a single interaction, causing a nucleotide base change (24). This applies to MNU and ENU, and their linear dose-responses suggest that there is no safe level of exposure (*in vitro* at least) for these and similar acting genotoxins (i.e., those that predominantly generate adducts at O atoms). However, this concept should now be reviewed as compounds that primarily alkylate N^7-G, and rarely O atoms show nonlinear responses, as we have shown human cells can cope with an insult from MMS and EMS at doses below our identified LOEL concentrations. Due to the analogous responses observed for MMS and EMS, these data have the potential to be extrapolated to other agents with similar reactive mechanisms and/or DNA targets.

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